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Transfer of triclocarban from mother to offspring during periods of development

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Introduction:

It has been shown that a variety of chemicals are not removed after wastewater treatment, which results in their release back into environment and water supply. These chemicals may act as endocrine disrupting compounds (EDC), which may affect the function of the endocrine system and adversely affect progeny. Studies evaluating the effects of EDC during periods of offspring development are lacking, including quantitative measures of accumulation after exposure. These EDCs have the potential to bioaccumulate and transfer from mother to offspring during periods of development. This includes both gestational and lactational exposure; many EDCs are highly fat soluble therefore, transfer in breastmilk during lactation may be a major route of exposure to offspring (Diamanti-Kandarakis et al, 2009). EDC exposure during periods of development has been associated with detrimental effects including changes in neurological development, behavior and reproductive abnormalities, and even obesity (Falconer et al, 2006). However, the data for many EDCs are limited; chronic exposures, which more accurately mimic the exposure from contaminated drinking water, have not been done. These studies are needed to investigate the potential detrimental effects after exposure.

Triclocarban (TCC) is an antimicrobial found in many personal care products including deodorants, antibacterial soaps and toothpaste. Given its widespread use, TCC is found as a contaminant in drinking water with concentrations ranging from pM to nM. Evidence of endocrine disruption after TCC exposure has been shown; when given to rats with 3-trifluoromethyl-4,4'-dichlorocabanilide (TFC) in their chow, their offspring showed a lower testes/body weight ratio (Nolen and Dierckman, 1979). Other studies have shown that TCC can interact with endogenous hormones to potentially affect development of sex organs and interfere with reproduction (Ahn et al. 2008, Chen et al. 2008, Huang et al 2014).

In this work, we are investigating the transfer of TCC from mother to offspring during periods of gestation and lactation. The transfer of TCC from mother to offspring was quantified using accelerator mass spectrometry (AMS). The exceptional sensitivity of AMS (pmol-amol) allows for quantification of very small quantities of ^{14}C- labeled material (Vogel 1995). In addition to its high sensitivity, the long half-life of ^{14}C ($t_{1/2}$=5730yrs) allows for long term tracking of material. Taken together, these characteristics allow us to investigate the transfer of environmentally relevant concentrations of TCC from mother to offspring over long periods of time.

Methods:

Chemicals

^{14}C-Triclocarban (TCC) was purchased from Moravek Biochemicals (Brea, CA). Specific activity of TCC was 30mCi/mmol. TCC was dissolved in dimethyl sulfoxide

(DMSO) prior; the percent in drinking water was less than 0.001%. Water aliquots for dose determination were added to Universol cocktail before scintillation counting.

Cell Culture

The BeWo b30 placental choriocarcinoma cell line was obtained from the lab of M. Saunders (Bristol Initiative for Research of Child Health, Bristol, UK) with permission from Alan Schwartz (Washington University, St Louis, MO). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine-Penicillin-Streptomycin, 1% Amphotericin B, and 1% MEM non-essential amino acids. Cells were maintained in polystyrene cell-culture flasks at 37 °C in 5% CO_2. At confluence, cells were sub-cultured using trypsin-EDTA.

Transwell Assays

1.12 cm^2 transwell inserts with 0.4 μm pores were coated with human placental collagen and presoaked in PBS. 112,000 cells were then seeded on the insert in 0.5 mL cell culture media with 1.5 mL cell culture media in the basolateral chamber. The cells were maintained in culture medium and monitored for the formation of a confluent monolayer by both light microscopy and measurement of the trans epithelial electrical resistance (TEER). In order to measure the transport of ^{14}C-TCC, the compound in HBSS was added to the apical chamber of a transwell insert with a confluent monolayer of BeWo b30 cells. At each timepoint, 100 μL was removed from the basolateral side containing 1.5 mL of HBSS. 100 μL of HBSS was then added back to the basolateral side of the transwell filter. AMS was used to determine the pmoles of ^{14}C-TCC transferred to

the basolateral chamber. Correction was done for the ^{14}C-TCC removed at each previous time point according to the method used by Poulsen et al. The apparent permeability coefficients for the doses of TCC were calculated using the following equation used by Carreira et al.:

$$P_{app} = k * V_w / A * 60$$

Where P_{app} is the apparent permeability in cm/s, k is the transfer rate constant calculated from the slope of the graph of time versus percent of initial dose in min^{-1}, V_W is the volume of well sampled in mL, and A is the diffusion area in cm^2.

P_{app} was background corrected by performing the transport assay using wells coated only with placental collagen to determine the permeability coefficient, P_e, using the following equation from Carreira et al.:

$$1/P_e = 1/P_{app} - 1/\text{ Blank } P_{app}$$

Bottle Preparation and Stability

Custom made water bottles (n=2) were tested for stability with 100nM ^{14}C-TCC over two weeks with liquid scintillation counting. A glass control was tested for comparison. An aliquot from each bottle was counted at the initial time point (1 hour) and each subsequent time point; data is expressed as percent of activity lost. Based on the average loss observed (~30%), 130nM ^{14}C-TCC water bottles were prepared for all

exposures. Bottles were stabilized for two days before dosing; once administered to animals, bottles were changed every 4-5 days. For each dosing bottle, an aliquot was taken before and after administration and counted with liquid scintillation counting (Perkin-Elmer Tri-Carb 2810 TR liquid scintillation analyzer). While in use, bottles were weighed to estimate water consumption by each dam. Both consumption amount and activity measured by scintillation counting were used to estimate administered dose.

Animals

All animal experiments were conducted following the guidelines and regulations set by Lawrence Livermore National Laboratory, including IACUC approval. CD-1 female (8-10 weeks) and male mice (10-12 weeks) were used for all studies (Harlan). Mice were housed individually in cages containing hardwood bedding and kept on a 12 h light/dark cycle in a ventilated room. Food and water were provided *ad libitum*.

^{14}C-TCC dosing and sample collection

For TCC exposures, females were mated with male mice (4 females, 1 male per cage). Figure 1a and 1b illustrate the experimental design for both *in utero* and lactation exposure studies, respectively. At each collection time point, offspring were euthanized, weighed, and rinsed three times in 1X PBS before storing at -80°C for AMS analysis. For *in utero* exposure, dams (n=6) were given ^{14}C-TCC drinking water starting on the day of plug visualization through gestation day 18 (GD18). At GD18, fetuses were separated from the maternal and fetal placentas, rinsed three times in 1X PBS and stored at -80°C for analysis. Placental tissue, once isolated, was rinsed and stored similarly to fetal tissue. For the lactation exposure group, dams (n=8) were given standard drinking

water until litters were born (Day 19/20). On the day of litter delivery, ^{14}C-TCC drinking water was given to dams for the first 10 days of lactation. On post natal day 10 (PND10) offspring were collected, rinsed and stored for analysis. For each exposure group, control dams with standard drinking water were run in parallel for comparison (gestation n=6, lactation n=2).

AMS analysis

Tissues were homogenized prior to analysis using a previously established method (Malfatti et al. 2012). Samples were incubated in 1-2ml of digestion buffer overnight at 37°C with gentle agitation; after digestion, samples were vortexed to break up the tissue in solution. Plasma samples were analyzed neat, no digestion was necessary. A small aliquot of each sample (10-100 µl, depending on tissue carbon content) was used for analysis. Prior to AMS analysis, samples were converted to graphite as previously described (Creek et al. 1997). The resulting $^{14}C/^{12}C$ ratios obtained by AMS were converted to pmol of TCC/g of tissue or ml of plasma after subtraction of the background carbon contributed from the sample, and correction for the specific activity, and the carbon content of the sample (10-15% for tissue, 3.8% plasma) (Malfatti et al. 2012).

Results and Discussion:

TCC transfer across the placental barrier was investigated (Figure 1) using a human placenta cell culture model system in which the BeWo immortalized cell line was utilized. The BeWo cell line has been used to investigate maternal to fetal exchange (Rytting and Audus, 2008) and has been shown to yield similar results to the placental

perfusion assay (Poulson, 2009). For our three doses tested (0.1nM, 1nM and 10nM), less than 10% of the dose was transferred from the maternal (apical) to fetal (basolateral) chamber. Based on these results, we moved on to *in vivo* exposures; we chose a 100nM TCC dose. Given the small amount of transfer observed (<10%) for the in vitro assay, we chose to investigate a higher, environmentally relevant dose *in vivo*. For these exposures custom made water bottles needed to be developed for the ^{14}C-TCC dosing. Up to 76% of the ^{14}C-TCC was found to adsorb onto materials (plastic, rubber) used in the traditional water bottles for rodents. We modified Klean Kanteen water bottles with mouse sippers; for these bottles, ~30% of the dose was lost after two days (Figure 2). To accommodate for this loss, we prepared 130nM drinking water as the dose for these studies.

Transfer of triclocarban from mother to offspring was investigated in two separate experiments that focused on placental and lactational transfer (Figure 2a). We found that TCC transfers through both exposure routes as shown in Figure 2b. Levels of TCC were detected in both fetal (~0.011%ID/g) and maternal (~0.007%ID/g) placental tissue, indicating transfer across the placental barrier had occurred. TCC was also present in fetal tissue at GD18 (%0.005%ID/g). For the lactation exposure, a significantly higher concentration was found in offspring tissue at PND10 (0.015%ID/g, $p<0.001$). This increased transfer was expected; TCC is lipophilic, therefore, we expected a higher amount of transfer to occur through the lactational route compared to the placental barrier. An interesting finding after both exposures was the average weight for offspring (Figure 2c). We observed a significant increase in offspring weight for both TCC exposure groups when compared to their control counterparts. After gestational exposure, TCC offspring were significantly ($p<0.05$) greater in body weight (1.26g ± 0.03)

compared to the control group (1.17g ± 0.04). A greater significance ($p<0.001$) was found for the lactational exposure group; TCC exposed offspring at PND10 were 5.72g ± 0.10 compared to the control group (4.30g ± 0.17).

To the best of our knowledge, this is the first report of quantitation of TCC transfer from mother to offspring during periods of development. TCC is capable of transferring from mother to offspring through both gestation and lactation and these concentrations are associated with increases in offspring weight, which may have implications for obesity. We are currently completing a long term study based on this data to investigate whether this increase in offspring weight persists through maturity and whether there are changes in fat metabolism for these exposed offspring. Other known EDCs such as bisphenol A (BPA) and diethylstilbestrol (DES) have been shown to increase offspring weight in developing offspring and interfere with fat metabolism. Differences between exposed male and female offspring will also be determined.

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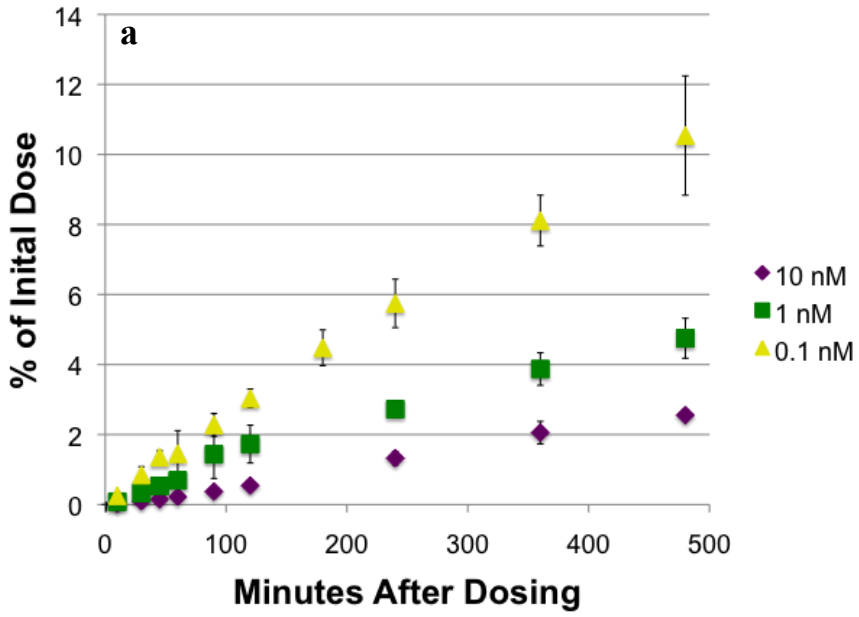


b

TCC Concentration (nM)	P_e (x 10^{-4} cm/s)
10	1.48
1	4.29
0.1	9.37

Figure 1. TCC transfer across the placental barrier *in vitro* a) Transfer of nanomolar ^{14}C-TCC in BeWo b30 cells over 8 hours b) Calculated permeability coefficients from transwell data shown in a).

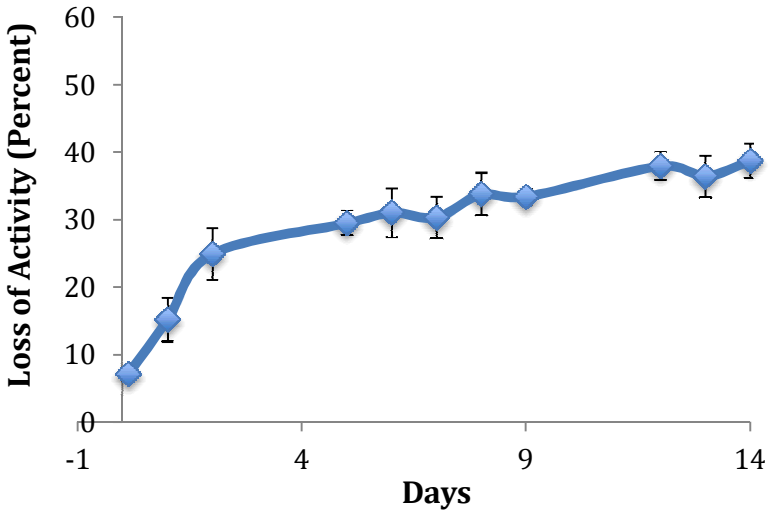
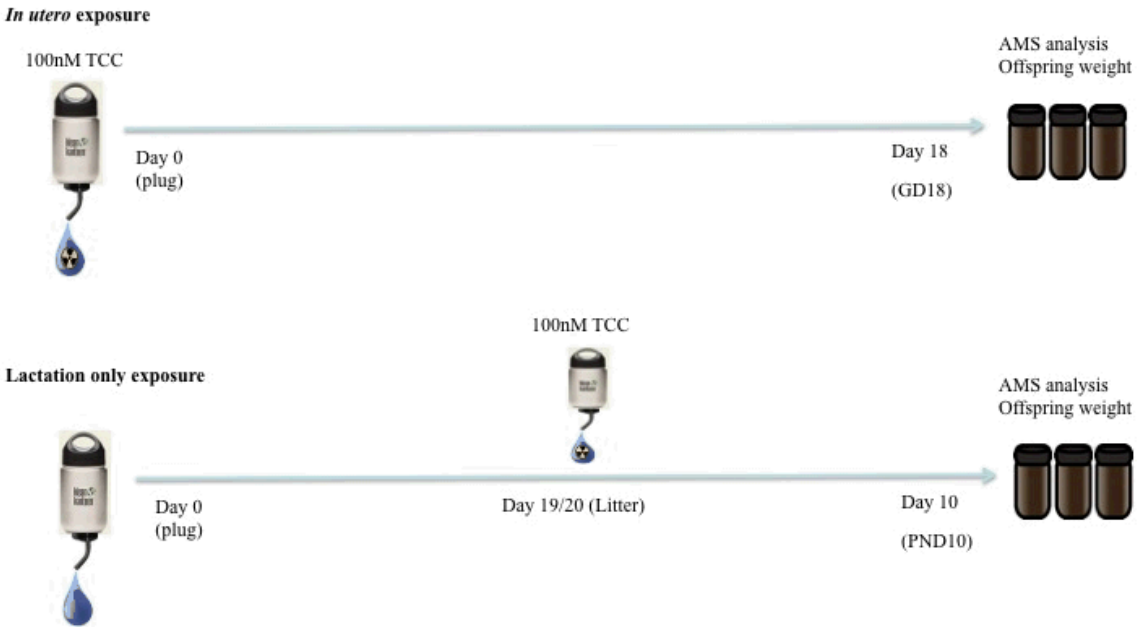
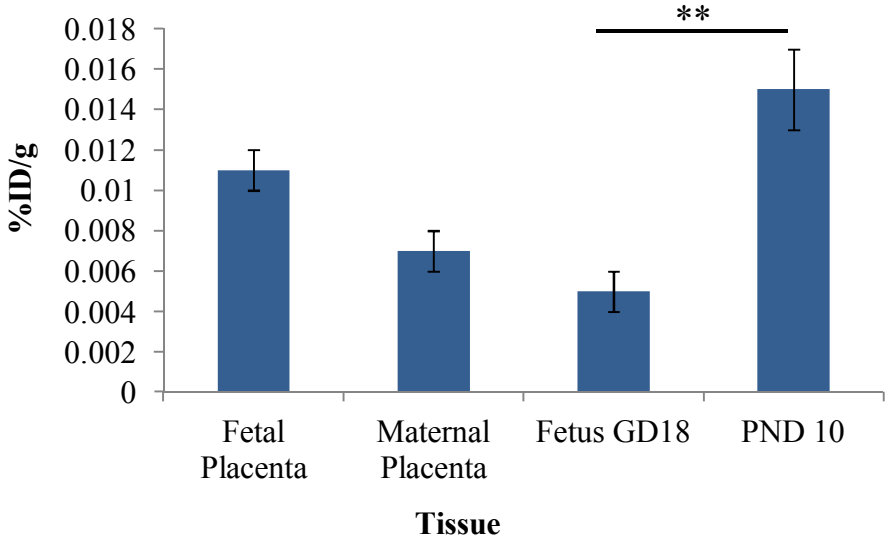


Figure 2. Custom made water stability over two weeks.

a


In utero exposure
100nM TCC
Day 0
(plug)
Day 18
(GD18)
AMS analysis
Offspring weight
Lactation only exposure
100nM TCC
Day 0
(plug)
Day 19/20 (Litter)
Day 10
(PND10)
AMS analysis
Offspring weight

b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0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
%ID/g
Fetal Placenta
Maternal Placenta
Fetus GD18
PND 10
Tissue

Group	Average weight (g) +/- SE
TCC-GD18	1.26 +/- 0.03*
Ctrl-GD18	1.17 +/- 0.04
TCC-PND10	5.72 +/- 0.10***
Ctrl-PND10	4.30 +/- 0.17

Figure 3. TCC exposure during gestation and lactation. Experimental design for gestation and lactation exposure routes shown in (a). Percent ingested dose per gram (%ID/g) in for gestation day 18 (GD18), placental tissue and postnatal day 10 (PND10) (b). Average offspring weight for GD18 and PND10 groups (c).